STUDIES ON THE INFLUENCE OF ANDROGENS ON THE REPRODUCTIVE PHYSIOLOGY OF THE GROUPERS

DISSERTATION SUBMITTED

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF FISHERIES SCIENCE

(MARICULTURE)

OF THE

CENTRAL INSTITUTE OF FISHERIES EDUCATION

(DEEMED UNIVERSITY)

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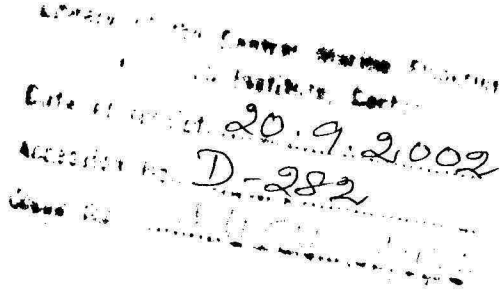

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ACKNOWLEDGEMENTS

I am greatly indebted to my supervising guide, major advisor, and chairman of dissertation advisory committee, **Dr. L. Krishnan**, Principal Scientist, Demersal Fisheries Division, CMFRI, for his guidance throughout the period of my dissertation. His daily observations, timely advice and stringent corrections helped in completing this work. My heart is always filled with gratitude for Co-chairman of the Advisory Committee, **Dr. T. Narender**, Scientist, Demersal Fisheries Division, CMFRI, for his inspiration and unlimited help. His directions, suggestions and support to formulate a palatable feed to the experimental fishes gave a right approach to the progress of this dissertation. I am really thankful to the member of my Advisory Committee, **Dr. K. K. Joshi**, Scientist (SS), Demersal Fisheries Division, CMFRI, for giving several useful tips on histology.

I express my deep sense of gratitude to the Director, **Prof.(Dr.) Mohan Joseph Modayil** for providing me all the facilities to carry out my dissertation in the most efficient way. The support, guidance and keen interest shown by **Dr. V. S. R. Murty**, Head, Demersal Fisheries Division, had enabled me to complete my dissertation in the most exemplary manner. I am really grateful to **Dr. Srinath,** Head, Fisheries Resources Assessment Division, for explaining to me the statistical set up of this experiment. I thank **Dr. Appukuttan**, Head, Molluscan Fisheries Division, for extending the facilities in his laboratory. I extend my deep sense of gratitude to **Dr. R. Paul Raj,** officer-in-charge, PGPM, who has enabled my access to all needful facilities in Physiology, Nutrition and Pathology Division.

I gratefully acknowledge **Thiru. P. Sampson Manickam**, Principal Scientist, Crustacean Fisheries Division for his timely suggestions in setting up the biofilters for this experimental study. I express my heartfelt gratitude to **Dr. K. C. George,** Principal Scientist, for his wonderful guidance in histology, photographing my slides and also for his useful tips to counter bacterial infections in the experimental fishes.

I express my gratitude to **Dr. Sunil Kumar Mohamed**, Senior Scientist, Molluscan Fisheries Division, for readily obliging to scan my histological slides. I remember with gratitude **Dr. A. G. Gopalakrishnan**, Scientist, NBFGR for his suggestions and inspiration throughout my dissertation. I am highly indebted to **Dr. (Mrs.) K. Shobana** for her suggestions on the maintenance of experimental fishes and also for her help in controlling the bacterial infections. I remember with gratitude **Mrs. Joselin Jose,** Scientist, Crustacean Fisheries Division, in helping me to procure the materials to set up the biofilters. I am really thankful to the director of Medivision diagnostic center and in particular **Dr. Antony**, pathologist, for his timely help in providing me the histology sections.

I whole-heartedly thank **Mr. Venugopal**, Technical Assistant, Fisheries Harbour Laboratory, Thoppumpady, for setting up the biofilters in the experimental tanks. I thank **Mr. Nandakumar Rao**, Techical Assisstant, Physiology, Nutrition and Pathology Division, for helping me in preparing the feed for this study. I thank in a very special way **Mr. Sathish Sahayak** for helping me in scanning the histological studies and also being a source of inspiration. I am really grateful to **Mr. Surendran**, Techical Assisstant, Physiology, Nutrition and Pathology Division for his guidance in histology. I sincerely thank **Mr. Rudhramoorthy** for the help and kindness that he had rendered to me. I thank **Mr.Tippuswamy**, Technical Assisstant Physiology, Nutrition and Pathology Division for his constant encouragements. I am thankful to **Mr. Anil**, Technical Assisstant for his co-operation in carrying out this study.

I express my gratitude to **Mr. Subodha Kumar Patro,** SRF, for helping me to transport the live animals and to **Mr. Bose,** Technical Assisstant, for providing me the live groupers for this experimental study. I thank in a special way **Mr. Sasidharan** and **Mr. Shaji** for maintaining the fishes in my absence. I am really grateful to **Mr. Chandrasekhar Rao and Abraham** Ph.D Scholars and **Miss. Liya Ambipillai** for their help in typing the manuscript. I express my sincere thanks to **Mrs. Nisha** and **Mr. Babu** Research Scholars for the help in preparation of this manuscript.

I wholeheartedly thank **Mr. Edwin** and **Miss. Seema** for analyzing the various water quality parameters of the experimental tanks. My thanks are also due to **Miss. Smitha** and **Mr. Susanta Behera** for their help in histology. I thank Mr. **Mohamed Koya** and **Mr. Vijayakumar** of 2001-2003 M.F.Sc batch for their kind help in the transportation of fishes.

I am thankful to my family members for their continuous encouragement and whole hearted support throughout my study.

Finally I thank Director, CIFE, ICAR, for having awarded me the fellowship during the study period.

सारांश

ग्रूपर मछलियाँ स्त्रीपूर्वी उभयलिंगी मानी जाती है. प्राकृतिक वातावरण में नर मछलियों का अनुपात मादाओं की अपेक्षा बहुत कम है. इनकी सब से छोटी जाति *इपिनेफेलस डयाकान्थस* का लिंग विपर्यय इस अध्ययन का आधार है. आहार में पुरुष होर्मोन आन्ड्रोजन का मिलावट करके खिलाते हुए निरीक्षण चलाए थे. होर्मोन का मिलावट मछली के भार के अनुसार किया था याने कि एक कि ग्राम भार केलिए पहले ग्रूप केलिए 0.5 मि ग्रा और दूसरे ग्रूप केलिए 1.0 मि ग्राम की मात्रा में. इन मिलावटों की मात्रा से परीक्षण में कहने योग्य व्यतियान नहीं देखा गया था जबकि 80 दिवस के अन्तराल में किए गए निरीक्षणों ने दिखाया कि ये मछलियाँ हार्मोन मिश्रित खाद्य से खिलाने से 25 वां दिवस में लिंग विपर्यय करती हुई 40 वें दिवस में मादा से नर बन गईं. फिर भी वृषणों का प्रकार्यात्मक परिपक्वन नहीं हुआ था. पर होर्मोन का मिलावट न करके समान खाद्य से खिलाई मछलियाँ इसी अन्तराल में मादा ही रही थी.

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ABSTRACT

Specimens of *Epinephelus diacanthus*, a smaller sized protogynous grouper were experimented to study the process of sex reversal by feeding diets having two different concentrations (0.5mg and 1.0mg / kg body weight) of the androgen 17α-Methyl testosterone for a period of 80 days. Morphological and histological studies showed that both the diets irrespective of the concentration of the androgen initiated sex reversal even on the 25th day of the experiment. The reversal of sex from female to male was complete on the 40th day of the experiment. The gonads remained in the same stage on the 60th day and 80th day of the experimental period. However functional maturity of the testes was not achieved. In control groups of fishes which were fed with the same diet without hormone, fishes remained as females in immature stages.

CONTENTS

LIST OF TABLES

Page. No.

LIST OF FIGURES

Page. No.

List of Plates

Page No.

PREFACE

Among the fishes, the only hermaphrodites are teleosts. The male and female tissues coexist in the gonad of hermaphroditic fishes. In this coexistence, either the spermatogonia or the oogonia is kept active and proliferating; while the other remains dormant; sometimes both remain synchronously active also (detailed examples and description follow in the succeeding chapters). The occurrence of hermaphroditism in fishes may be of evolutionary significance, enhancing their propagation. Hence the study of hermaphroditism in fishes is ever interesting for a fish biologist.

Groupers (*Epinephelus sp*) are one among the several genera in which hermaphroditism has been studied and reported in detail by early fish biologists. In recent times considerable interest has been shown in the cage culture of these fishes. Fast growth, good meat quality and the accrued monetary benefits are the main reasons behind grouper culture. In South East Asian countries like Hong Kong, Singapore, Malaysia, and Thailand, groupers are much preferred among other food fishes. *E.akaara* is served as a delicacy in marriage feasts in Hong Kong.

Groupers are protogynous hermaphrodites i.e., they mature as females and later convert their sex into males. This change in sex is critical in broodstock management, especially regarding the maintenance of adequate numbers of male spawners. Acceleration of the process of natural sex reversal seems to be a better alternative to get functional males. Sex reversal of groupers can be induced by social control, feeding hormone and environmental manipulation. Among these, hormonal sex reversal is more practical.

Induced sex reversal of the blue-spotted grouper, *Epinephelus fario* and greasy grouper, *E. tauvina* was achieved by oral administration of 17-α Methyl testosterone. For the first time in the country, the CMFRI, Cochin has successfully achieved sex inversion and natural spawning in *E. tauvina* in the captive conditions (Dr.Grace Mathew, personal communication).

However, further studies are required to understand the transition of sex from the female to male in groupers fed with androgens. Considering the importance of this aspect the present study was conducted on *E. diacanthus,* since the size is small among other groupers as ideal size, which is commercially exploited by trawlers along the west coast of India.

1.INTRODUCTION

1. INTRODUCTION

Fishes exhibit wide diversity in sexuality such as gonochorism and hermaphroditism. 'Gonochorism' is the existence of separate sexes, which is the normal mode of sexuality as in higher animals. In 'hermaphroditism', an individual can be male and female simultaneously or successively in its lifetime. Atz (1964) has defined hermaphroditism as the existence of recognizable male and female tissues in the gonads of an individual. Three basic forms of hermaphroditism are reported in fishes; protogynous hermaphroditism in which individuals develop first as females and turn later into males e.g. groupers, protandrous hermaphroditism in which the male stage differentiates first e.g. sea bass, and synchronous hermaphroditism where both male and female stages coexist functionally eg. *Serranus scriba.* In both protandry and protogyny generally the largest animal in the population transforms into the opposite sex. It is generally assumed that the diverse sex patterns such as intersexuality and sex reversal in the teleosts is related to the lack of cortical and medullary organization in the embryonic gonad. According to Yamamoto (1969) the reason for hermaphroditism is that apart from the superior sex genes in the Y and X chromosomes, multiple sex factors existed in the autosomes. In exceptional individuals the superior sex genes in the sex chromosomes might be overridden by many opposing autosomal genes resulting in XX males or XY females.

The genus *Epinephelus* belonging to the subfamily Epinephelinae and family Serranidae, includes large food fishes commonly known as groupers, rockcods, hinds and locally as 'kalava'. Most frequently groupers are found in hard-rocky bottoms (hence the name rockcods) within depth of 100 meters and extending up to 200 meters. The habitats of these fishes vary from coral reefs, estuaries and rocky bottoms. Groupers are demersal fishes inhabiting the tropical and subtropical waters of all the oceans. So far thirty eight species of groupers have been reported from the seas around India (Heemstra and Randall, 1993). Groupers fetch good price in the market, hence it forms an important component of the fisheries of the tropical and subtropical nations.

Groupers are protogynous hermaphrodites i.e., they mature as female and later transform their sex into male. Several species of groupers such as *E.tauvina, E. malabaricus, E. fario* are candidates for aquaculture because of their high growth rate, environmental tolerance and palatability in South East Asian nations such as Thailand, Hong Kong, Taiwan etc. Lack of stability in sex poses severe problem in brood stock development, concerning mainly the availability of adequate male spawners for fertilizing the eggs. To retain our aquaculture interests over the hermaphroditic fishes it is desirable to control their sexes. However the phenotypic expression of sex in teleosts is complex and cannot be generalized for all the members. It is agreeable that chemical substances called sex inductors (may be steroids) play an important role in the control of sex in both gonochoristic and ambisexual fishes.

By administering androgens (17-α Methyl testosterone, testosterone, androstenedione etc.) orally, it is possible to overcome the above limitations in female groupers. These hormones accelerate the process of sex reversal from female to male in the protogynous hermaphrodites. Steroid treatments have been used successfully in several gonochoristic species in order to develop mono sex population (either male or female) in fish culture (reviewed by Hunter and Donaldson, 1983) where monetary benefits are involved. Success in induced sex reversal in *E. tauvina* at the age of 3 years by oral administration of 17-α Methyl testosterone (17MT) through the feed was reported by Chen *et al.*(1977). In *E. fario* sex inversion was achieved at the age of 2 years by the same hormone.

A study of sex reversal in the species mentioned above requires the maintenance of a large number of animals and it is particularly difficult because of their huge size and expenditure involved in their maintenance.

Even though it is generally believed that the androgens result in the expression of male sex alone; reports of paradoxical feminization are not uncommon (Nakamura, 1975). Such paradoxical feminization is very often attributed to high dosages of hormone. Hence in this study efforts have been made to assess an appropriate dosage of hormone, which induces the sex reversal of groupers and its

accompanying histological changes in the gonad as well as related aspects i.e., gonadosomatic index (GSI) using a smaller sized species, *Epinephelus diacanthus*, which is equally captured in huge quantities in trawls and hook and lines along the Indian coast. *E. diacanthus* is generally called spinycheek or thornycheek grouper and these fishes occur at a depth of 40-100 meters along the west coast of India.

2.REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The most diverse display of sexuality is prevalent among the class Pisces. Fishes exhibit various patterns of sex ontogeny by which their gonad becomes a functional ovary or testis. Sex reversal or hermaphroditism is rare among the elasmobranchs, ganoids and the lungfishes. Smith (1975) has critically reviewed the occurrence of hermaphroditism in fishes. More recently Reinboth (1988) has given an insight to the physiology of consecutive hermaphrodites.

A great variety of functional hermaphroditism, both normal and abnormal has been found in various taxonomic groups of the teleosts. According to Atz (1964) normal hermaphroditism refers to those that exist in a uniform way at some time during the ontogeny of all or many members of a species. Chan and Yeung (1983) had further stated that a deviation from the normal types of hermaphroditism might not always imply sterility, as some abnormal hermaphrodites were in fact functional. Testicular tissue has been observed by Spurway (1957) in the ovaries of the normally gonochoristic guppy, *Poecilia reticulata.* The occurrence of spermatozoa had been reported from the ovotestis of a hermaphroditic salmon. (Atz, 1964).

Normal hermaphroditism with or without sex reversal is known to occur in a number of teleost orders. Protandrous hermaphroditism has been reported in Ostariophysi, Polynemidae, Scorpaeniformes (Platycephalidae) and probably in Stomiatiformes. Protogynous hermaphroditism is found in Synbranchiformes. Synchronous hermaphroditism has been found in Aulopiformes and Antheriniformes (Chan and Yeung, 1983). Several types of functional hermaphroditism are found in Perciformes.

Smith (1959) had studied several members of the family Serranidae from the waters of Bermuda. He concluded that members of the genera *Prinodus* and *Hypoplecturus* to be a synchronous hermaphrodites. Among the serranids the genus

Epinephelus is considered to be protogynous hermaphrodite. Some sparids like *Taius tumifrons* and *Spondyliosoma cantharus* are protogynous hermaphrodites while *Sparus auratus, S. longispinis* and *Rhabdosargus sarba* are all protandrous hermaphrodites. Some sort of rudimentary hermaphroditism has also been reported from sparids like *Boops boops* and *Diplodus vulgaris*. *Dentex dentex*, another member of the Sparidae family is a true gonochorist. Robertson (1972), Shen and Liu (1976) and Moyer and Nakazono (1978) have reported about the extensive occurrence of protandry and protogyny among the coral fishes of the families Scaridae and Labridae.

Hermaphroditism from Indian waters was reported by Chacko and Krishnamoorthy (1949) in the Indian shad, *Hilsa ilisha;* Prabhu and Antony Raja (1959) in the Indian mackerel, *Rastrelliger kanagurta;* Nayak (1959) in *Polynemus heptadactylus;* Raju (1960) in the Skipjack, *Katuswonous pelamis;* Antony Raja (1963) in the Oil sardine, *Sardinella longiceps;* Hida (1967) in Polynemids, Patnaik (1967) in the Indian salmon, *Eleutheronema tetradactylum;* Kagwade (1969) in *Polydactylus indicus* and Dorairaj (1973) in the Threadfin fish, *Polynemus microstoma*.

The diverse sex patterns generally encountered in the teleosts may be explained by the lack of cortical and medullary organisation in the embryonic gonad (Chan and Yeung, 1983 and Guraya, 2000).

Dodd (1983) had studied the process of sex differentiation among elasmobranchs. He stated that sex differentiation in elasmobranchs essentially consists of the proliferation of cortex and regression of the medulla in the females and *vice-versa* in males. The regressive part (cortex in the male, medulla in the female) rarely differentiates further than the indifferent gonadal stage. The onset of sex differentiation from the indifferent gonadal stage cannot be made out in elasmobranchs (Dodd, 1983).

In teleosts the sexually indifferent stage may last for several years as observed in the European eel, *Anguilla anguilla* (Chan and Yeung, 1983). The central idea of Guraya (2000) that the primordial germ cells of the indifferent gonad giving raise to male and female germ cells needs careful consideration. The primordial germ cells divide into male/ female germ cells under the influence of some sex indicators (Witschi, 1934, 1957; D'Ancona, 1949, 1950 and Yamamoto, 1962).

Pandian and Koteeswaran (1998, 1999) were of the view that gonad differentiation is influenced by growth factors which are directly under the control of sex steroids.

The process of sex differentiation in the consecutive hermaphrodites is as follows: juvenile hermaphrodite→ functional female→ intersex→ functional male (in groupers). The gonad of a hermaphrodite bears clearly recognizable male and female tissues (Atz, 1964). D'Ancona (1950) believed that the gonads in teleosts should be considered as undifferentiated, even when oocytes are present because male gonocytes may not have differentiated.

From the beginning of differentiation of the gonads in ambisexual fish, it contains both spermatocytes and oocytes (Yamamoto, 1969). Observation by Debas *et al.* (1989) revealed that the intersex phase of the protogynous grouper, *Epinephelus microdon* resembles a return to the immature female phase indicating sex differentiation in progress.

Guraya (2000) believed the differentiation of the gonad to be under the control of external and internal factors affecting the fish and also under the genetic factors.

The genetic basis of the sex-reversing hermaphrodites is poorly understood; however some sort of genetic basis may prevail. The universal xx-xy or zz-zw systems of sex determination are not well established in sequential hermaphrodites (Chan, 1970). Moe (1969) ruled out the possibility of genetic basis of

sex determination in *Epinephelus morio*, a sequential hermaphrodite; while Warner (1975) argued that the capacity of an animal to function ambisexually is genetically programmed.

Several theories governing the role of chemical factors in sex determination have been proposed (Witschi, 1934, 1957; D' Ancona, 1949, 1950 and Yamamato, 1962). Chemical substances like 'androgenine' and 'gynogenine' (D' Ancona, 1949, 1950) 'gynotermone' and 'androtermone' or 'gynoinductor' and 'androinductor' (Yamamoto, 1962) have been proposed. Even though such chemical substances have not been isolated they are generally believed to be homologous with steroids. The male specific antigen H-Y was pointed out to play a determinative role in the evolution of sexes (Wachtel and Koo, 1981).

Pandian and Koteeswaran (1998, 1999) were invariably of the view that gonad differentiation is influenced by growth factors (proteins) which are directly under the control of sex steroids while Reinboth (1980, 1985) strongly believed that the change in steroidal profiles as the major cause for sex inversion in teleosts.

Experimental evidence of Chan *et al.* (1975) supported the idea of chemical substances determining sex. He claimed gonadotropins (LH) to be responsible for sex inversion in the protogynous, *Monopterus*.

Warner *et al.* (1975) reported that more rapidly growing fishes change sex sooner than other individuals of the same age and fish that grow slowly may not change sex. Moe (1969) felt that the process of sex reversal is more connected with the spawning activity than with the age or growth of a species. Moe (1969) mentioned that sex reversal takes place "under conditions of over crowding and food scarcity, numerous individuals may be induced to transform during the critical ages of 7- 12 years. Since each transformation is equivalent to the death of the female, the effect of transition may be sufficient to alter the reproductive potential of the population."

Frequent sex reversions (secondary males converting into females) observed by Debas *et al.* (1989) suggest that some form of social control exists among protogynous hermaphrodites. The sex inversions and reversions may go on for a period of time until the sex ratio reaches 1:1 (Debas *et al.*, 1989).

Yamazaki (1976) underlined the essential role of the fish endocrine system and other nervous and genetic factors in controlling the gonad differentiation and maturation.

The normal pattern of gonadal development in teleosts can be changed with the administration of hormones or by other suitable physiological disturbances (Fostier *et al.*, 1983). Experiments conducted on some gonochoristic species (*Oryzias latipes*) revealed the capacity of steroids to control sex (Yamamoto, 1969). The administration of oestradiol to the larvae produced 100% females in Yamamoto's experiments. The successful experimentation of steroids over gonochorists had raised hopes of such sex control in ambisexual fishes.

In his experiments with the silver eel D' Ancona (1948) could successfully sex reverse the differentiated male by administering estradiol. The susceptibility of teleostian gonad to chemical substances in general and sex steroids in particular appears certain.

Chan and Philips (1969) and Colombo *et al.* (1972) had observed a higher androgen to oestrogen ratio *in vitro* in the male phase of *Monopterus albus* and *Sparus auratus*. Attempts were made by Debas *et al.* (1989) to correlate the sex steroid profiles with the various stages of sexuality in the protogynous grouper *E. microdon*. He encountered similar levels of testosterone and estradiol in all types of sexualities (female - intersex and male). The intersexual stage of the fish appeared to be characterised by a decrease of 11-ketotestosterone and a rise of 11β-hydroxy androstenedione (Debas *et al.*, 1989).

In general groupers lack secondary sexual characteristics. As they are protogynous, it can be assumed that sex is a function of age and size (body length and weight). Debas *et al.* (1989) adopted weight as a better criterion because of its versatility to distinguish presumptive sexes. The age and size at which the groupers undergo sex reversal vary with the species.

Renu *et al.* (2002) reported natural sex reversal of *Epinephelus malabaricus* in 26 month old individuals and functional males were 34 months old weighing around 5.0 Kg. *Epinephelus tauvina* matured at 3 years and sex reversed at 7 years of age (Tan and Tan, 1974).

Sex reversal has been controlled hormonally (Yamamoto, 1962), environmentally (Harrington, 1971) and socially (Fishelson, 1970; Robertson, 1972 and Ross, 1990).

Successful results of induced sex reversal by means of oral administration of Methyl testosterone (MT) for several consecutive months have been reported for *E. tauvina* (Chen *et al.*, 1977), *E.akaara* (Tseng and Ho, 1988) and *E. fario* (Kuo *et al.*, 1988).

E. malabaricus weighing more than 5.0 kg may have some part of their ovaries changing to testicular tissues already. These fishes could be induced to male broodstock by using 17-α Methyltestosterone (Pakdee and Tantavanit, 1985; Julavittayanukul *et al.*, 1985;Rattanachot and Pakdee, 1986; Chen *et al.*, 1977 and Kuo *et al.*, 1988). The suitable time to induce sex reversal is out of spawning season, so that functional males will be obtained in the next spawning season.

Tessy (1994) had reported that *Epinephelus diacanthus* matured mostly around 166 mm SL; undergo sex inversion from 34 month onwards measuring 230mm-310mm in standard length. The occurrence of males increased progressively as one moved above 270mms suggesting sex reversal in progress.

At a particular point of time in the natural population the occurrence of female sex outnumbers male in groupers. This is because apparently the gender of an animal is a function of age/ size, suggesting larger males and smaller females.

The idea of sex steroids determining the sex of an individual is still accepted (Reinboth, 1980, 1985). Hence it is widely believed that by increasing the relative concentration of androgen/ estrogen in an undifferentiated, intersex species the desired sex can be produced.

Studies on the cell and molecular biology of development and sex differentiation of fish gonads are of great current interest because sex steroids added to the water or food are found to reverse or sterilize the gonads of the fry, either because one sex has greater economic value or because high population densities produce smaller or less valuable fish (Hunter and Donaldson, 1983).

Endocrine control of sex differentiation was initially conducted on ornamental fishes like the Medaka, *Oryzias latipes* in 1950's and 1960's (Yamamoto, 1969). Sex steroids (androgens/ estrogens) are useful in obtaining the desirable sex in tilapia and salmons (Donaldson, 1996).

Currently male tilapia is produced by the oral administration of 17 α-Methyl testosterone at a rate of 30 – 60 mg/Kg feed to newly hatched tilapia fry (7 – 12 days old, 9 – 11 mm TL, 10 – 15 mg total weight). Feeding with 17 α - Methyl testosterone for a period of 21 – 28 days result in 95% males (Shelton *et al.*, 1978; Tayamen and Shelton, 1978). Feeding of the fry is synchronized with period of sex differentiation and done only till the 28th day (Bartholomew, 2000). Administration of 17 α - Methyl testosterone to produce all -male population is the most practicable technique (Pandian and Varadaraj, 1987).

The desired hormonal action depends mainly on the efficacy of the steroid, as well as the dose, method of administration and time and duration of treatment for the tilapias (Shelton *et al.*, 1978). The dosages of hormone is very

critical in sex control, as low doses of androgen induce masculinization and high doses result in paradoxical feminization (Piferrer and Donaldson, 1991), and high doses if administered over a long period of time may result in sterilization (Piferrer *et al.*, 1994) of salmon alevins.

Piferrer and Donaldson (1991) had compared the efficiency of several androgens. They claimed that testosterone is ineffective in inducing masculinization, than 11- ketotestosterone which is very effective. The non- aromatizable synthetic androgen 17α- Methyl testosterone is capable of inducing masculinization without paradoxical feminization (Piferrer and Donaldson, 1991). However paradoxial feminization was observed in some cichlids treated with 17α- Methyl testosterone (Nakamura, 1975).

As groupers are protogynous, the male grouper can be obtained by accelerating the process of sex reversal of bigger matured females by oral application of methyl testosterone at a dosage of 1mg/Kg for a period of about 2 months (Ratanachot and Pakdee, 1986 and Ruangpanit *et al.*, 1988). Kuo *et al.* (1988) had reported successful sex reversal and spermiation of under 2 year old *Epinephelus fario* (blue spotted grouper). Mature males were obtained successfully by feeding 17α- Methyl testosterone at 0.5 mg/Kg and 1.0 mg/Kg. However implants of testosterone in *Thalassoma bifasciatum* failed to induce sex reversal of females (Kramer *et al.*, 1988).

The above review will show that experimental studies on sex reversal of groupers in India are very few.

3.MATERIALS AND METHODS

3. MATERIALS AND METHODS

The fishes, *E. diacanthus* (plate 1) that occur fairly in good numbers in the Cochin area formed the study material for this experiment. The fish samples were collected from the catches landed at the Fisheries harbour; Murukumpadam, Cochin (India) and live fishes were collected from the trawl boats operated along the Kerala coast by local fishermen.

3.1 COLLECTION OF FISHES FROM WILD AND THEIR OBSERVATION

The smaller sized dead specimens (ranging from 118 - 214 mm. SL) of *E. diacanthus* were collected and examined in detail to study the existence, shape and arrangement of the gonads. Local fishermen, who were onboard the trawlers were clearly instructed to bring fresh and properly iced specimens to avoid chances of degradation.

The fish sample brought to the harbor were transferred into an icebox and then carried to the CMFRI hatchery complex for further studies. In the laboratory the total length (TL), standard length (SL) and the corresponding weight (W) of the specimens were measured. After recording the morphometric characters, the fishes were dissected out with a sharp scalpel. The visceral organs were pushed apart to observe the presence of the underlying gonad. After examining the gonad, a small piece of it was cut and teased over a slide to observe the sex of the animal (Guerrero and Shelton, 1974) under the microscope.

3.2 COLLECTION OF LIVE ANIMALS

E. diacanthus occur mostly at depths of 40 m along 9° 46.18' N and 75° 59.05' E off Cochin. Fishes were brought from the trawlers having a Length Overall (LOA) of 15.6 m and a beam of 4.5 m. The trawlers were fishing mainly for anchovies and sardines in April. Each fishing trip lasted for 36-48 hours.

Plate 1. *E.diacanthus* used in the experimental study.

Plate 2. Experimental setup of the present study

The fishermen, who were specially briefed about the task, sorted out live *E. diacanthus* that occurred in the trawls, and healthy, uninjured fishes were selected and transferred into a 200 L (0.78 x 0.50 x 0.51m) FRP tank, filled with sea water. The tank water was aerated by battery-operated aerator. Before leaving the fishes into the tank their swim bladder was punctured with a sharp sterile needle because these fishes live in greater depth and under pressure. The fishes were maintained in the FRP tanks for nearly 36-48 hrs while the trawlers were sailing towards the landing centre at the Murukkumpadam fishing harbour of Cochin. The arrival of the trawler at the fishing harbour was communicated through a mobile phone to our team. From the Harbour the live fishes (approximately 60 Nos.) were transported by the Institute's jeep in the same tank with aeration to the Hatchery complex of the CMFRI, wherein their length and weight were recorded. They were given a formalin dip treatment for 5 minutes at a concentration of 250 ppm., before releasing them into the experimental tanks (1 control and 2 experimental tanks).

3.3 SETTING OF BIOLOGICAL FILTERS IN THE TANKS

The experimental tanks used were of 5 tonne capacity,rectangular in shape and made up of FRP. The tanks had a length of 2.5 m, breadth of 1.6 m and height of 1.5 m. Four such tanks were set side by side in the experimental site.

The experimental tanks were provided with built in biological filters. Cuttlefish bones, charcoal, gravel and river sand were used to set up biological filters. Cuttlefish bones were taken and washed thoroughly to remove the adhering meat particles. The shell pieces were then sun-dried to a crispy texture. The Cuttlefish bones were then broken into smaller pieces and stored in containers until further use. Charcoal, baby metal granite pieces and river sand were also thoroughly washed to remove all the adhering dirt, mud and clay particles. The washed materials were then sun-dried and stored in gunny bags for preparation of the filters.

Perforated PVC pipes of 1.25" diameter were arranged diagonally over the bottom of the experimental tanks with airlift pipes emerging from the corners. Air

diffusers were suspended inside the airlift pipes to bubble out the filtered low ammonia containing seawater.

Over the diagonally arranged pipes, calcareous shells (cuttlefish bones, oyster shells and some clam shells) were filled to a height of 16 cm, followed by a layer of charcoal to a height of 6 cm. A mosquito net was spread above the charcoal layer. Dry Granite pieces of ¼" diameter was spread over the mosquito net till a height of 6cm.This layer was again sealed off with mosquito net to accommodate a forthcoming layer of river sand to a height of 6 cm.

Seawater from Manasseri was brought with the help of tankers and stored in 30 t RCC tanks of the hatchery complex for 10 days. The settled, sediment free seawater was pumped inside the experimental tanks for housing the fishes.

The water in the tanks was kept under circulation for nearly a month to mature the filter bed. In each tank 12 fishes were released, having approximately equal length and weight distribution (106-184 mm. SL and 50-120 gm) respectively. Apart from the three experimental tanks one tank was set aside to house the remaining fishes for further studies. The tanks were named according to the hormone concentration fed to the fishes.

Control - MTO

0.5 MG – MTO

1.O MG- MTO

All the tanks were covered with a dark cloth to avoid excess light and the fishes were acclimatized for a week to the hatchery conditions. The fishes were also trained to take formulated feed.

3.4 FEED FORMULATION

A formulated feed was prepared for the groupers with the locally available ingredients as given below:

INGREDIENTS	PERCENTAGE(%)
Fish meal	25
Shrimp meal	20
Squid meal	10
Ground nut oil cake	12
Rice bran	8
Wheat flour	20
Tapioca flour	4.5
Cod liver oil	0.5
Total	100.0

All the ingredients excluding the Cod liver oil were thoroughly mixed well using a Mixer. Tap water of 80 ml was sprinkled over the components and finally mixed well to make into a dough. The ingredients were cooked in free flowing steam for 15 minutes inside a pressure cooker and the ingredients were left undisturbed after cooking. In the meantime 5 gms of agar (bacteriological grade) and 1 gm of gelatin (bacteriological grade) were mixed thoroughly in 80 ml of tap water and boiled well for 5 min. The molten agar cum gelatin was poured over the cooked dough and mixed thoroughly. Agar and gelatin was used as binders. Cod liver oil was added finally. The feed was prepared for every 15 days.

The quantity of MT to be added to the feed was calculated by taking into account of the actual biomass of the experimental fishes in each group. The biomass was recalculated at frequent intervals to account for the changes caused due to sample removal.

3.4.1 Incorporation of 17α- Methyl testosterone

The food thus prepared was divided into three parts to mix the hormone (17∝ methyl testosterone) in different concentrations (0.5 mg/kg body weight and 1mg/kg body weight). The Methyl testosterone used for this experiment was purchased from Acros Organic laboratories, Belgium.

3.4.2 Feed of 1 mg. 17MT concentration

The biomass of the fishes to be fed with 1.0 mg MT conc. was 955gms. The requirement of MT was calculated for each day of feeding and accordingly feed for 15 days was prepared. For this 0.955 x 15 mg of 17 - α Methyl testosterone was weighed carefully in Mettler balance (microgram accuracy) and then mixed well with 25 ml of rectified spirit. The alcohol dissolved methyl testosterone was then atomized (to one portion of feed) to homogeneously distribute into each particle of the feed.

3.4.3 Feed of 0.5 mg. 17MT concentration

The biomass of the fishes to be fed with 0.5 mg MT conc. was 1230 gms. The requirement of MT was calculated for each day of feeding and accordingly feed for 15 days was prepared. For this 0.615 x 15 mg of 17-αMethyl testosterone was weighed in a Mettler balance and mixed well in rectified spirit. The resultant mixture was atomized (to the second portion of feed) using a sprayer over the dough.

3.4.4 Control - MTO

The biomass of the fishes to be fed with control feed was 1100 gms. The control feed (the third portion) was sprayed with an equal quantity of rectified spirit with a sprayer.

The feed in the form of dough was then dried inside an oven at 60^0Cto evaporate the sprayed alcohol. After drying the feed, everyday ration was weighed separately in a series of polythene bottles and labeled to mark the respective tanks to be fed. The bottles were stored in a deep freezer at -8^0C. The estimated moisture content of the formulated feed was 44%.

3.5 FEEDING

Everyday the feed was taken out of the freezer and kept outside for some time to reach the room temperature. The feed taken out was rolled into strands of approximately 3-4 mm diameter and broken down into small pieces to be fed to the respective tanks. Fishes were fed at 2% of their body weight. Feeding was done regularly at 1030-1100 hrs daily according to the fish biomass, except on Sundays. The quantity of feed given was recorded daily.

3.6 MAINTENANCE OF WATER QUALITY

Salinity and temperature of the sea water in the experimental tanks was checked once a day at peak hours (1500 hrs). Salinity of the experimental tanks was maintained at 32 ppt., throughout the experimental period (April 2002 to June 2002).

Water quality parameters like dissolved oxygen, ammonia and pH were checked once in a week. Dissolved Oxygen (DO) was estimated using modified Winkler's method; ammonia (NH_3) was estimated by phenol hypochlorite method (Zolarzano,1969) and pH was checked by a pH meter (Mettler Tolledo) .The optimum water quality followed for the maintenance was as follows:

Salinity - 32 ppt.

Dissolved oxygen - >6.0 ppm

Ammonia - <0.01 ppm

pH -7.8±0.2

3.7 SAMPLING PROCEDURE/ PROCESSING

On preplanned, periodical intervals from the start of the experiment, two fishes from each tank, were caught randomly and sacrificed to assess the effect of the androgen 17-αMT on the sex-reversal of the fishes. The following parameters were taken before sacrification:

Total Length.

Standard Length.

Whole weight

Eviscerated weight

Length between nose and operculum

Length between dorsal and pelvic fins (after Debas *et al.*, 1989)

The weight of the gonad was noted down to calculate the Gonadosomatic index (GSI). The gonads were then finally fixed in Bouin's fluid for histological studies. Fixation was carried out for 24 hrs.

The fixed gonads were taken and washed thoroughly in running tap water to remove traces of picric acid. Dehydration of the tissues was done in ascending grades of alcohol 50%, 70% and 90% and finally given three changes in absolute alcohol for 6 hrs.

Clearing was carried out for three hours in Chloroform with two changes. Finally the tissue was impregnated with molten wax at 60^0C for 6 hrs with three changes. Finally the tissues were embedded in paraffin.

The sections were cut at thickness of 5 μm in a rotary microtome. Sections were stained with haematoxylin-eosin and mounted in DPX. The slides were observed under light microscope to see the gonadal changes.

3.8 EXPERIMENTAL PROTOCOL

The main objective of the experiment was to study in detail the sex reversal of the immature female *E.diacanthus* into male by oral administration of 17α - MT using two dosages of 17α - MT to find out the optimal dosage, required for complete sex reversal. To achieve the objective, the experiment was arranged as follows:

The groupers maintained in the tanks were sacrificed periodically to study in depth, the change in gonad morphology and histology and to assess the progress in sex inversion. Experimental fishes were sampled on the 25th, 40th, 60th and 80th day from the start of the experiment, as described below:

As a preliminary study on the 25th day one fish from each tank was sacrificed. For subsequent histological observations (on the 40th and 60th day) 2-3 experimental fishes of uniform sizes from each of the experimental tanks were caught in a hand net carefully and transferred to 25 litre volume buckets. Finally on the 80th day only fishes from the treatment tanks were sacrificed to assess the changes in gonadal histology. The buckets were marked Control-MTO, 0.5 MG-MTO and 1.0 MG-MTO, to identify fishes appropriately.

The fishes caught were killed by a sudden blow over their head. The morphometric characters were then carefully recorded. The fishes were dissected to separate the gonads and they were scanned under dissection microscope (10X, Leica model) to understand the morphology. A piece of gonad was fixed in Bouin's fluid for histological studies. The histological slides of the gonadal samples from the two fishes were compared to the results of the gonads.

The classification by Kuo *et al.* (1988) was followed to study the gonadal developmental classes of the sacrificed experimental fishes.

Gonadal development classes	Description
1.	Gonadal tissue primarily of female germinal cells, predominantly previtellogenic oocytes. This stage includes chromatin-nucleus and peri-nucleolus stages.
2.	Oocytes at yolk vesicle stage appear, but those at peri-nucleolus stage predominate in the ovarian tissue indicating the commencement of vitellogenesis.
3.	Oocytes at the late phase of yolk vesicle predominate in the ovarian tissue. However yolk granule (vitellogenin) deposition in oocytes is evident.
4.	Ovarian tissues fully developed and consist of oocytes at yolk globule stage. The ovary is notably enlarged and GSI exceeds 2.0
5.	Ovary consists of oocytes at various phases of vitellogenesis, but atresia of vitellogenic oocytes is evident to varying extent.
6.	Beginning of sex transition, primary oocytes remain; spermatogonia begin to appear among the ovarian tissue.

7.	Artesia of primary oocytes and spermatogenesis are both progressing, testicular tissue becomes the dominant constituent of the gonadal structure.
8.	Large numbers of spermatogonia and spermatocytes but a few oogonia are present.
9.	Testicular development in the resting phase of spermatogenesis; few oogonia are evident.
10.	Spermatogenesis progressed to spermatids and spermatozoa phase. The tissue shows strong basophilic nature.
11.	Testis is greatly reduced in size and the spermatozoa ejaculated.

4. RESULTS

4. RESULTS

4.1 ENVIRONMENTAL PARAMETERS

In the present experiment on the grouper, *E. diacanthus*, the environmental parameters observed during the study period (April 2002 to July 2002) are as follows. The temperature ranged from 28.2 - 30.5°C in the experimental tanks. The dissolved oxygen ranged between 6.2 ppm - 7.1 ppm. Ammonia ranged between 0.01 to 0.18 ppm and p^H was found to vary from 7.65 to 8.16. The salinity was maintained always at 32 ppt.

4.2 OBSERVATIONS ON THE FEEDING BEHAVIOR OF *E.DIACANTHUS*

The well developed big eyes of the groupers is an indication that the groupers feed by sight. During feeding, the groupers congregate near the feeding spot and they eagerly devoured the feed offered.Majority of the groupers gulped the feed while in the water column itself.However some particles which settled at the tank bottom were also eaten by some fishes.

4.3 MORPHOLOGICAL AND HISTOLOGICAL DETAILS OF THE GONADS OF THE WILD COLLECTED GROUPERS (Table - I gives the details of the fishes collected from wild).

The freshly dissected gonads from the wild specimens (118 – 214 mm SL) were off-white in colour. The two lobes were clearly seen and were unequal in size. These gonads taken from the wild specimens were examined by the acetocarmine squash method (Guerrero and Shelton, 1974). The results showed all of the examined fishes to be females in the immature phase thus confirming the protogynous nature of *E. diacanthus*.

Table I				
Morphometric data collected from *E.diacanthus* for preliminary examination of sex using acetocarmine squash method:				
S I. No.	TL (mm)	SL (mm)	W (g)	Sex
1	244	199	176	♀
2	234	190	141	♀
3	209	170	94	♀
4	236	191	170	♀
5	253	205	173	♀
6	238	191	156	♀
7	226	183	122	♀
8	223	184	115	♀
9	218	178	109	♀
10	311	153	382	♀
11	279	118	298	♀
12	226	183	135	♀
13	256	191	145	♀
14	272	214	261	♀
15	281	126	280	♀

Where,

TL = Total length in mm

SL = Standard length in mm

W = Weight in gm

♀ = Female sex

The bilobed gonads were placed towards the posterior part of the abdominal cavity. The right and left lobes of the gonad were unequal in their sizes. The gonads were connected to the swim bladder, by the mesenteries and lie below it. The two lobes of the ovary join posteriorly and descend as oviduct and finally open to the exterior as genital pore.

4.3.1 Histology of the protogynous gonad

In histological sections the wall of the gonad is covered externally with a peritoneal layer. Longitudinal, oblique and circular muscle fibers were seen, within the tunica albuginea spread below the peritoneal layer. The lumen of the ovary is lined with germinal epithelium, which forms the surface layer of a series of folds or lamellae. The oocytes in their initial phase of development extend around the lamella (plate 3).

4.3.2 Maturity stages of the gonad of the wild stock

Histological sections of the gonads of the wild specimens revealed oocytes in the following stages of maturity.The gonads were in gonad development classification of class 1 stage (Kuo *et al.*, 1988).

4.3.3 Stage I of oocyte development

Stage I oocytes were in chromatin – nucleus stage. The oocytes measure 28 – 33 μm in size. The cytoplasm of the oocyte is strongly basophilic and had taken up stain unevenly. A large nucleolus is present in the centre of the nucleus. Nuclear margin is not clearly defined. Stage I oocytes predominated in the ovarian lamellae (plate 4).

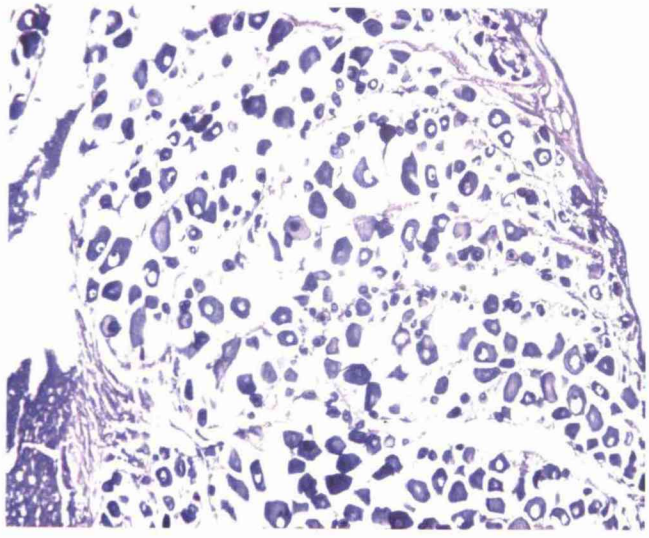


Plate 3. Histology of the *E.diacanthus* gonad (100x)

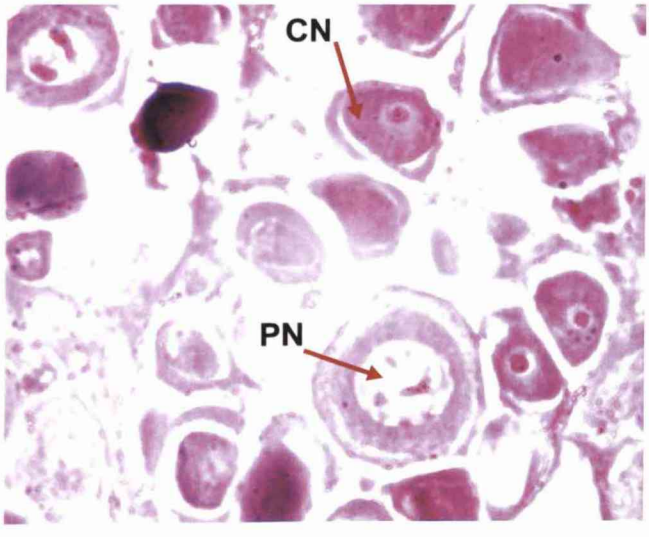


Plate 4. Maturity stage of the natural specimens (400x)
CN-Chromatin-nucleus, PN-Peri-nucleolus

4.3.4 Stage II of oocyte development

A very few stage II oocytes in peri - nucleolus stage were also seen in the maturing gonads. Their diameter ranged between 42 - 70 μm in size. More number of nucleoli were present in the perinucleolar stage and were arranged along the periphery of the nucleus. A follicular membrane surrounded the oocyte (plate 4).

Oocytes in the yolk vesicle stages were not seen. Stage I and Stage II oocytes were arranged along the periphery of the ovigerous lamella. The presence of stage I and stage II oocytes in the gonad of the wild specimens indicate that vitellogenesis had not commenced and that, wild specimens were immature at the start of the experiment.

4.4 MORPHOLOGICAL AND HISTOLOGICAL DETAILS OF THE GONADS OF THE EXPERIMENTAL FISHES SACRIFICED DURING THE Ist SAMPLING (25th DAY OF THE EXPERIMENT).

(Table II gives the details of sampling of the experimental fishes; Table III gives the relative amounts of hormone accumulated by the fishes through the feed.)

Control – MTO

On the 25th day, the gonad of the control diet fed fishes appeared small, but supple and rounded externally. The gonadosomatic index was 0.0340. The gonads were dull white and resembled the gonad of a wild specimen. In sections the ovarian lamellae were observed having clusters of developing oogonia. The cytoplasm of oogonium took light haematoxylin and nucleus stained brightly. The oogonia measured 8 μm. Clusters of oogonia were seen developing. The gonadal lamellae were seen clearly. The histology of the gonad revealed it to be in the immature stage. Oocytes in chromatin – nucleus and peri - nucleolus stages were absent. The observations revealed that the gonads were differentiating into ovaries and were in their primary stages of development (plate 5).

TABLE II

Morphometric data of experimental *E.diacanthus* used for histology.

Date of Sampling	Tanks	Sl.No	TL (mm)	SL mm	WW gm	E.W (gm)	L (N&OP)	L (D&P)	AVG. GSI(%)	
13. 05. 2002 I sampling (25th day)	Control-MTO	1	172	140	66	61	51	49	0.034	25th day
	0.5 MG-MTO	2	175	142	68	62	55	50	0.027	
	1.0 MG-MTO	3	182	146	64	61	56	50	0.03	
28.05.2002 II sampling (40th day)	Control-MTO	4	205	171	115	98	55	65	0.0605	40th day
		5	204	167	96	81	49	48		
	0.5 MG-MTO	6	215	183	168	136	56	75	0.0148	
		7	193	162	105	90	48	51		
	1.0 MG-MTO	8	192	158	110	91	47	55	0.0258	
17.06.2002 III sampling (60th day)	Control-MTO	9	182	149	96	85	47	60	0.0363	60th day
		10	210	170	111	98	64	61		
		11	193	159	104	97	56	55		
	0.5 MG-MTO	12	205	168	107	100	65	57	0.0197	
		13	217	178	129	118	65	64		
		14	210	173	144	127	62	62		
	1.0 MG-MTO	15	191	156	97	87	57	53	0.02145	
		16	193	159	100	88	58	55		
		17	200	165	115	100	60	57		
10. 7.2002 IV sampling (80th day)	0.5 MG-MTO	19	206	167	125	110	59	61	0.013	80th day
	1.0 MG-MTO	20	179	148	87	78	51	51	0.003	

Where

TL – Total length
SL – Standard length
WW – Whole weight in grams
EW – Eviscerated weight in grams

L(N&OP) – Length between nose and operculum
L(D&P) – Length between dorsal and pelvic fins
GSI – Gonadosomatic index.

Table III

Relative amounts of hormone accumulated through feed in the experimental *E.diacanthus*

Sampling	Control-MTO	0.5MG-MTO	1.0 MG-MTO
I Sampling 25th day	-	9.9 mg	19.1 mg
II Sampling 40th day	-	14.5 mg	29.1 mg
III Sampling 60th day	-	21.2 mg	44.8 mg
IV Sampling 80th day	-	30.7 mg	63.8 mg

Plate 5. Oogonia developing in the control gonad on 25th day (100x)
OG-Oogonia (Immature gonad)

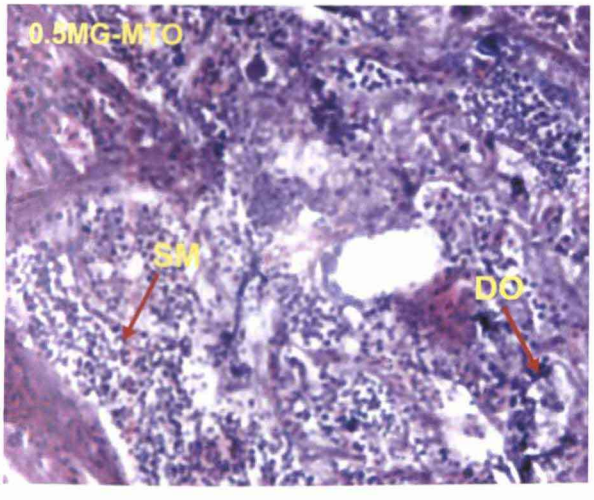


Plate 6. Degeneration of the 0.5MG-MTO gonad (400x) on 25th day
Gonadal class 7

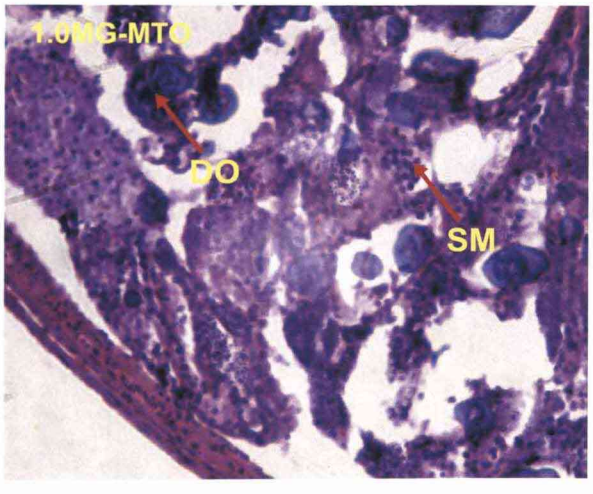


Plate 7. Degeneration of the 1.0MG-MTO gonad (400x) on 25th day
Gonadal class 7

DO-Degenerating Oocytes
SM-Spermatogonia

0.5 MG-MTO

The gonads of the experimental fishes looked thin and emaciated externally. The roundish appearance had changed and the color turned light pinkish. On the 25th day the total amount of 17-α MT administered, as calculated was 9.9 mg/kg body weight (BW). The gonadosomatic index was 0.027. A surprisingly clear degeneration of the female ovary to the testes was seen even in examinations under low power (100X). Most part of the gonad had regressed. Degenerating oogonia could also be observed, showing the process of sex reversal. Highly basophilic spermatogonia were seen developing in the gonads. A clear transformation of the female gonad to male testes was visible very clearly. In conclusion histological details showed very clearly the induction of the process of sex reversal even in a short period of 25 days of treatment The gonads were in gonadal development class 7 (vide., Kuo *et al.*, 1988)(plate 6).

1.0 MG- MTO:

The morphology of the gonads resembled the gonads of 0.5 MG-MTO.The gonads looked shrunken and narrow and were light pinkish in colour. On the 25th day the total amount of 17- α MT administered as calculated was 19.1 mg/kg body weight. The gonadosomatic index was 0.030. The gonadal transition from female to male as seen in 0.5 MG-MTO was observed here also. Highly basophilic spermatogonia and some developing spermatocytes were also seen. The gonads were in gonad development class 7 (vide., Kuo *et al.*, 1988) (plate 7).

In short, the gonads of 0.5 and 1mg treated fishes sampled on the 25th day looked similar histologically. The only difference was that the 0.5 MTO had very few regressing oocytes whereas in I MG- MTO comparatively more oocytes were seen.

4.5 MORPHOLOGICAL AND HISTOLOGICAL DETAILS OF THE GONADS OF THE EXPERIMENTAL FISHES SACRIFICED DURING 2nd SAMPLING (40th DAY OF THE EXPERIMENT)

Control- MTO

The gonad of the control group was off- white in colour, soft in texture and roundish in appearance. The gonad was slightly bigger in size (plate 8).

The mean gonadosomatic index of the fishes was 0.0605. The size of the oocytes ranged from 32- 42 μm. Oocytes were in chromatin nucleus and peri - nucleolus stages (plate 9). The developing oocytes were arranged along the periphery of the gonadal lamellae. The gonad was in the previtellogenic stage and had advanced a little in maturity.The gonad was in gonad development class 1 (vide., Kuo *et al.*, 1988)

0.5 MG- MTO

The gonads of the treated fishes were shrunken and appeared very thin (plate 10). The gonad was extensively connected by the mesenteries to the swim bladder. It was very difficult to identify and to separate the gonad from the swim bladder. Arrays of blood vessels emerging from various directions and flowing towards the gonads were seen in the abdominal cavity of the dissected fish. The periphery of the treated gonad was slightly pinkish white in colour; while the central portion of the gonad continued to be off white.

The amount of hormone fed to the fishes had risen to 14.5 mg/kg BW on the 40th day. The gonadosomatic index of the sacrificed fishes was 0.0145. Atretic oocytes were totally absent. Spermatocytes were observed and stained brightly with haematoxylin and exhibited clear margins (plates 12a and 12b). The sections of the gonad taken on the 40th day showed that sex reversal was almost complete even though functional reversal is yet to take place.The gonads were in the gonad development class 8(vide., Kuo *et al.*, 1988).

Plate 8. Roundish gonad of the control on 40th day (100x)

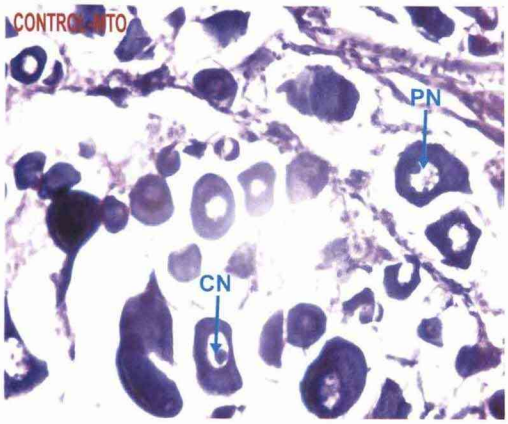


Plate 9. Oocytes developing in the control-MTO gonad (400x) on 40th day.

Gonadal class 1

Plate 10. Shrunken gonad of 0.5MG-MTO on 40th day (100x)

Plate 11. Shrunken gonad of 1.0MG-MTO on 40th day (100x)

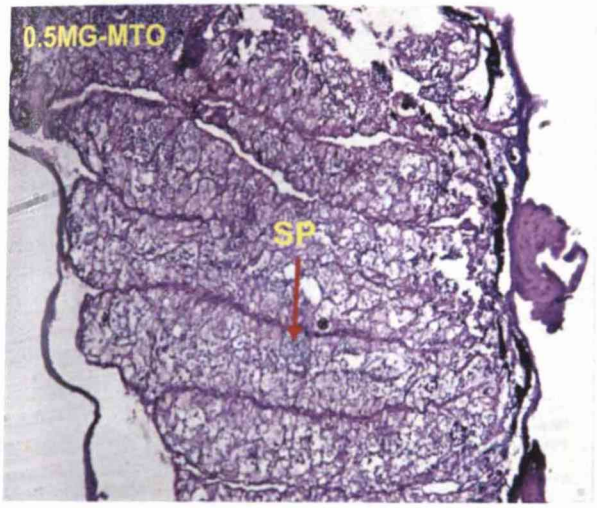


Plate 12 a. Spermatocytes developing in the 0.5MG - MTO gonad (100x) on 40th day.
SP-Spermatocytes

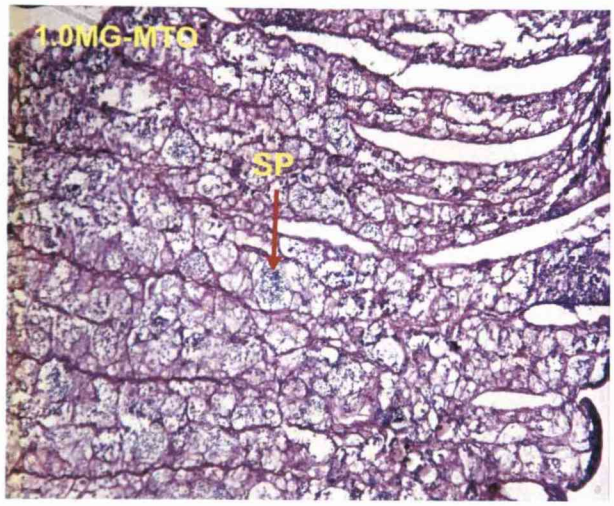


Plate 13 a. Spermatocytes developing in the 1.0MG - MTO gonad (100x) on 40th day.
SP- Spermatocytes

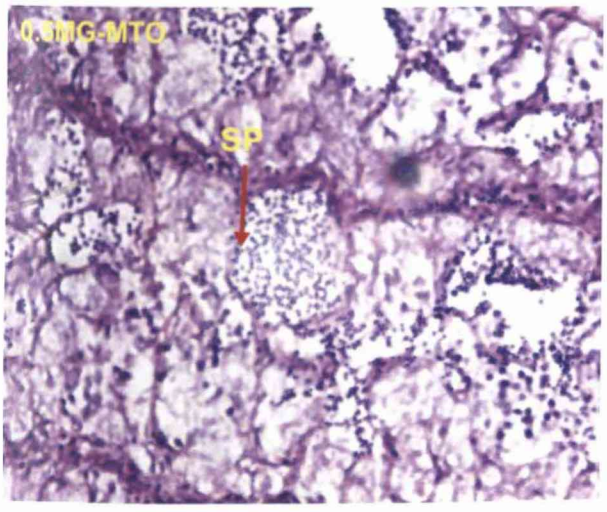


Plate 12 b. Spermatocytes developing in the 0.5MG-MTO gonad (400x) on 40th day.
SP-Spermatocytes

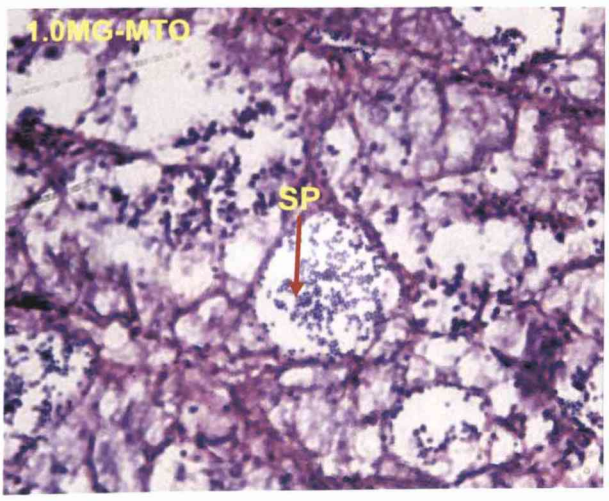


Plate 13 b. Spermatocytes developing in the 1.0MG-MTO gonad (400x) on 40th day.
SP-Spermatocytes

Gonadal class 8

1 MG-MTO

The gonads were shrunken, thin and resembled 0.5 MG-MTO gonads (plate 11). In this treatment also the separation of the gonad from the swim bladder was difficult.

On the 40th day the total hormone administered to the fishes was 29.1 mg/kg BW. The mean gonadosomatic index of the fishes was 0.0258. Spermatocytes had taken up haematoxylin uniformly and appeared reduced in their sizes, showing that possibly they are secondary spermatocytes. Clusters of spermatocytes could be seen (plates 13a and 13b). Atretic oocytes were absent. The gonads were in gonad development class 8 (vide., Kuo *et al.*, 1988).

4.6 MORPHOLOGICAL AND HISTOLOGICAL DETAILS OF THE GONADS OF THE EXPERIMENTAL FISHES SACRIFICED DURING THE 3rd SAMPLING (60th DAY OF THE EXPERIMENT)

Control – MTO

Control gonads were off white in colour and remained round and bulgy (see plate 14). They could be located much easily within the abdominal cavity; separation of the gonads from the swim bladder was also easier. Not much of a change was seen in the gonad on the 60th day. The gonadosomatic index of the sacrificed fishes was 0.0363. The gonad was in the gonad development class 1 (vide., Kuo *et al.*, 1988).

Histology of the gonads showed that oocytes were in the first and second stages of development as seen in the ovary observed on the 40th day. No further progress in oogenesis was observed.

Plate 14. Roundish gonad of the control on 60th day (100x)

Plate 15. Shrunken gonad of 0.5MG-MTO on 60th day (100x)

Plate 16. Shrunken gonad of 1.0MG-MTO on 60th day (100x)

0.5 MG-MTO

The morphology of the treated gonad was similar to that of the previously sampled fishes taken on the 40th day (plate 15). There was not any noticeable change in the morphology and appearance of the gonads. On the 60th day the total amount of MT administered as calculated was 21.2 mg/kg BW.The gonadosomatic index of the fishes was 0.0197.

A notable observation on the histology of the gonad in the specimen treated with 0.5 mg MT was the marked and progressive degeneration of the ovary. The degenerative changes were characterized with an increase in fibrous like material, an abundance of small eosinophilic cells and vacuolated areas. Such a tissue pervaded the entire gonad replacing areas formerly occupied by oocytes. An abundance of flattened and spindle shaped cells was visible. However spermatogenic tissue was also prevalent in less number of pockets. (plate 17). The gonad was in gonad development class 8 (vide., Kuo *et el.*, 1988)

1.0 MG- MTO

The morphology and histology of 1 mg treated gonad was very similar to that of the 0.5 mg- MT treated specimen (plate 16 and 18).

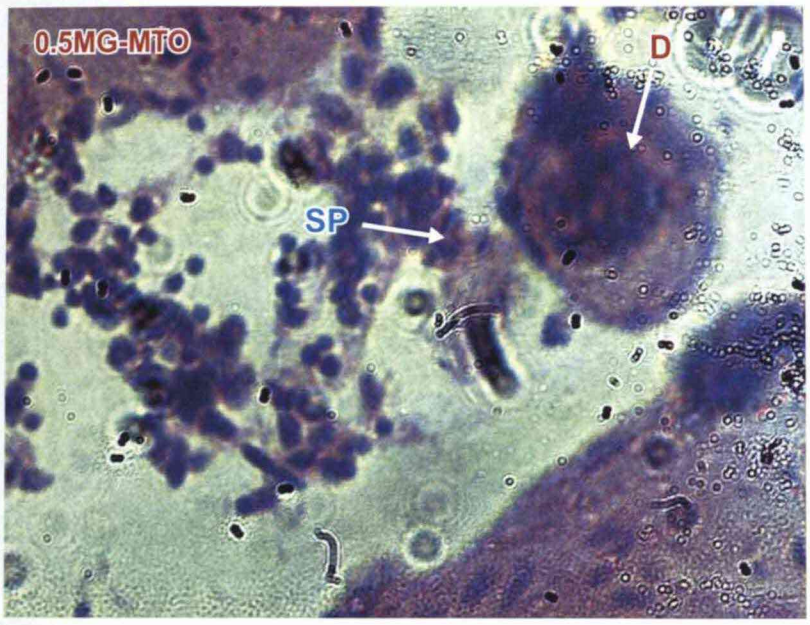


Plate 17. Degeneration of the 0.5MG-MTO gonad (280x) on 60th day
Gonadal class 8
SP-Spermatocytes
D-Degeneration of the ovarian tissue

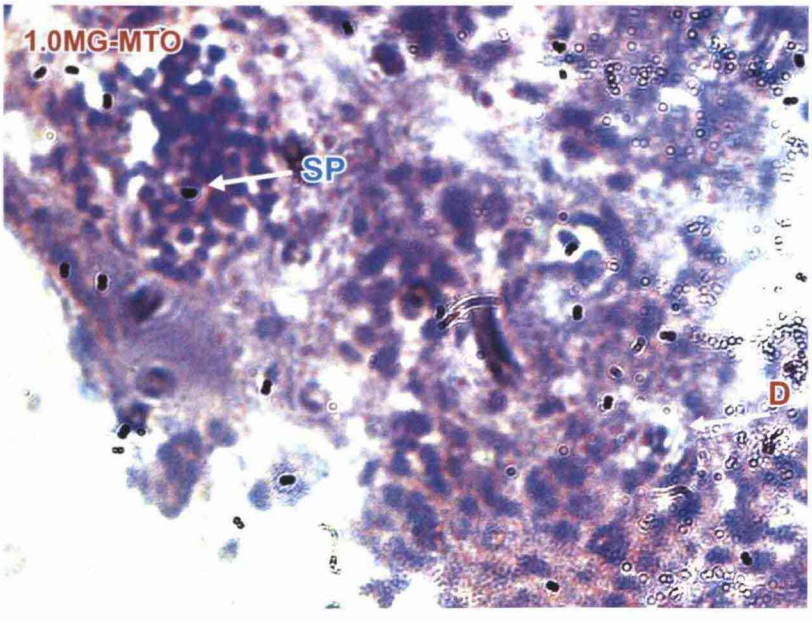


Plate 18. Degeneration of the 1.0MG-MTO gonad (280x) on 60th day
Gonadal class 8
SP-Spermatocytes
D-Degeneration of the ovarian tissue

4.7 MORPHOLOGICAL AND HISTOLOGICAL DETAILS OF THE GONADS OF THE EXPERIMENTAL FISHES SACRIFICED DURING THE 4th SAMPLING (80th DAY OF THE EXPERIMENT)

Before closing the experiments a few fishes from the 0.5mg and 1mg MT treated tanks were sacrificed and the gonads processed for histology. The details are given below:

0.5 MG-MTO

The gonads appeared shrunken and emaciated and were similar to that of the previous sampling. On the 80th day the MT fed to the experimental fishes as calculated was 30.7mg/kg BW.The gonadosomatic index of the fishes was 0.013. Histologically the gonad appeared almost similar to the gonad observed on the 60th day. The degeneration process greatly increased to such a point that the eosinophilic staining cells occupied along with vacuolated areas. Few pockets of spermatogenic tissues were also observed. (plate 19a and 19b). The gonads were in gonad development class 8 (vide., Kuo *et al.*, 1988)

1 MG- MTO

The gonad of the fish treated with I mg MT was exactly like the gonad treated with 0.5 mg MT.

However the observation to be stressed is that the functional maturity of the transformed gonad *i.e.*, testes had not been attained even on the 80th day. Spermatids and spermatozoa were absent and the spermatogenic activity was localized in small pockets (plate 20a and 20b). The gonads were in gonad develoment class 8 (vide., Kuo *et al.*, 1988).

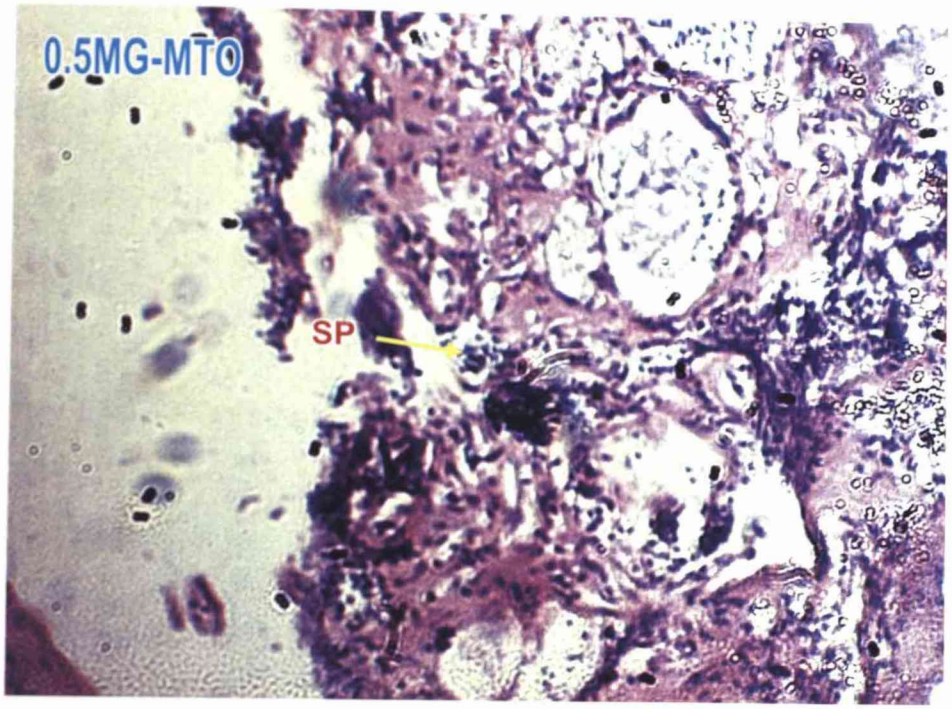


Plate 19 a. Degeneration of the 0.5MG-MTO gonad (70x) on 80th day
Gonadal class 8
SP-Spermatocytes

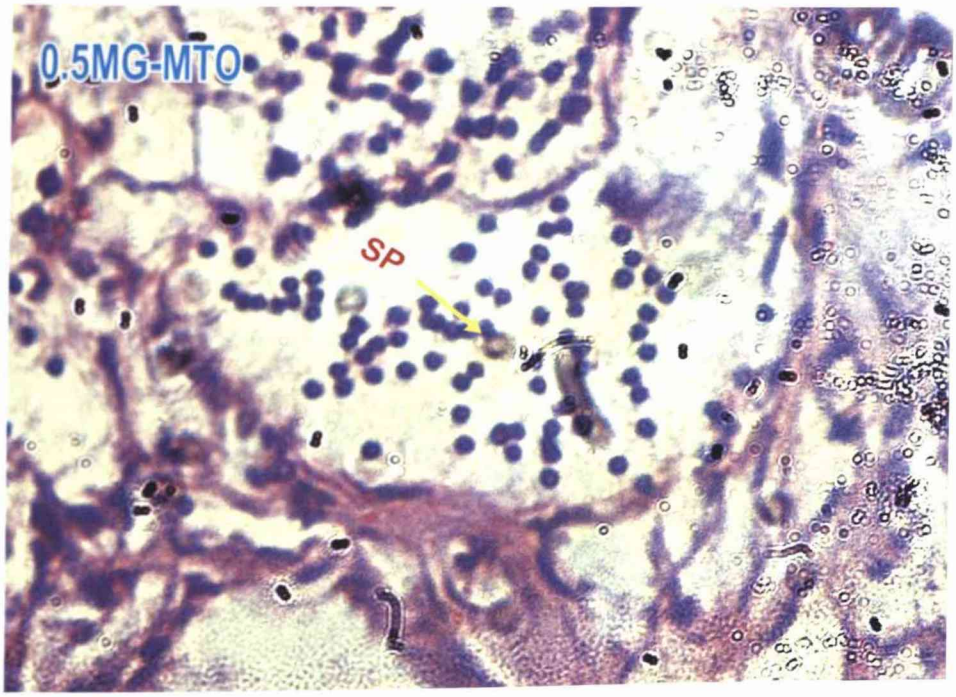


Plate 19 b. Spermatocytic activity in the 0.5MG-MTO gonad (280x) on 80th day
Gonadal class 8
SP-Spermatocytes

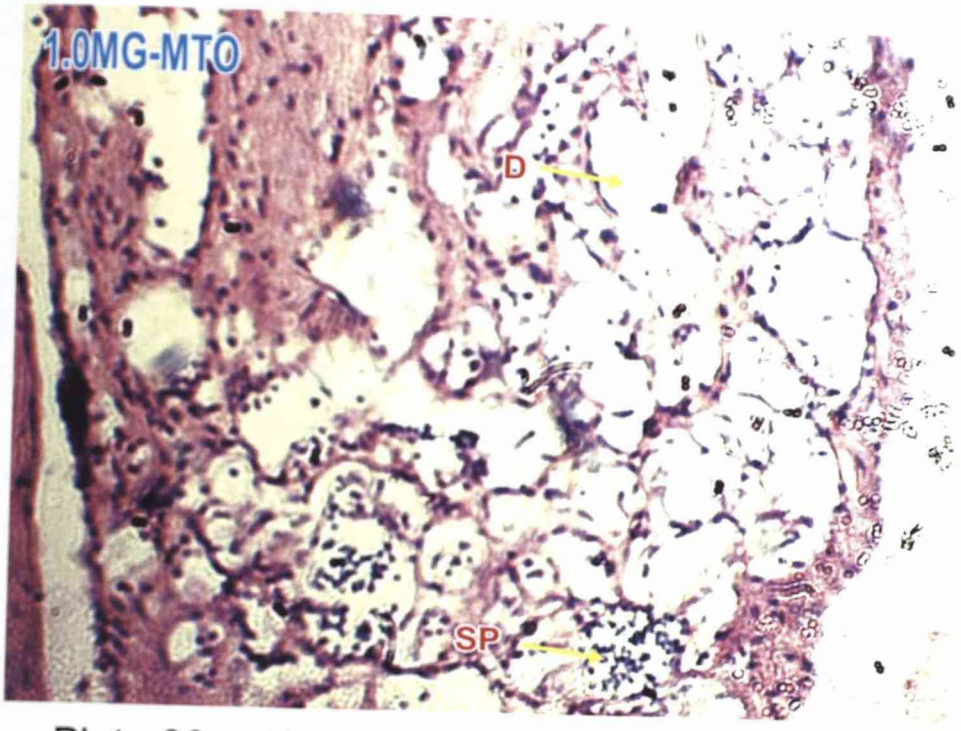


Plate 20 a. Degeneration of the 1.0MG-MTO gonad (70x) on 80th day
SP-Spermatocytes

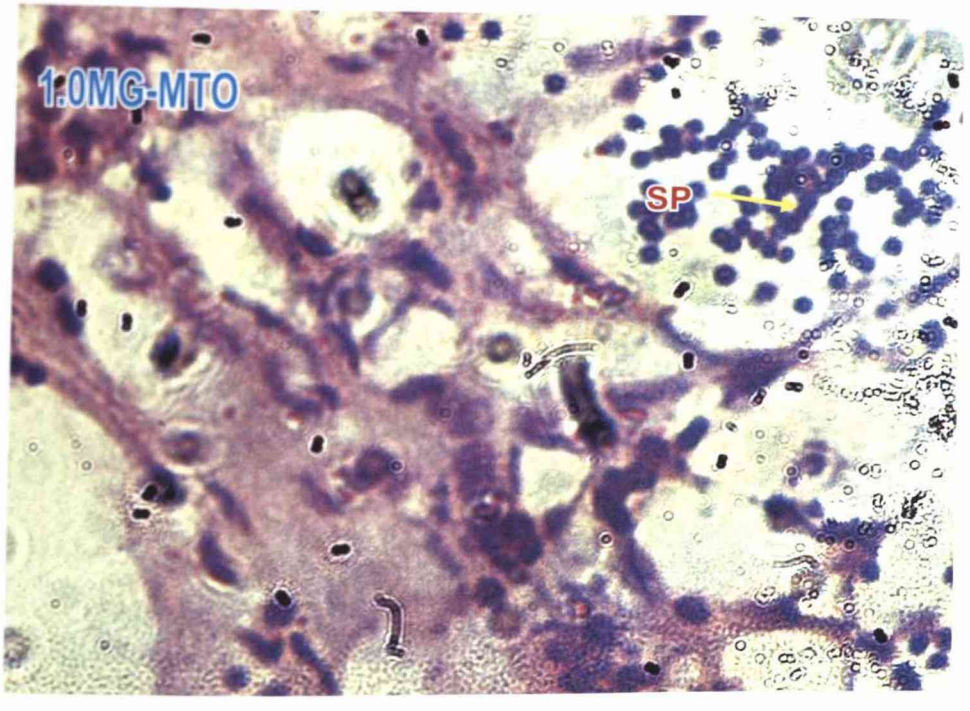


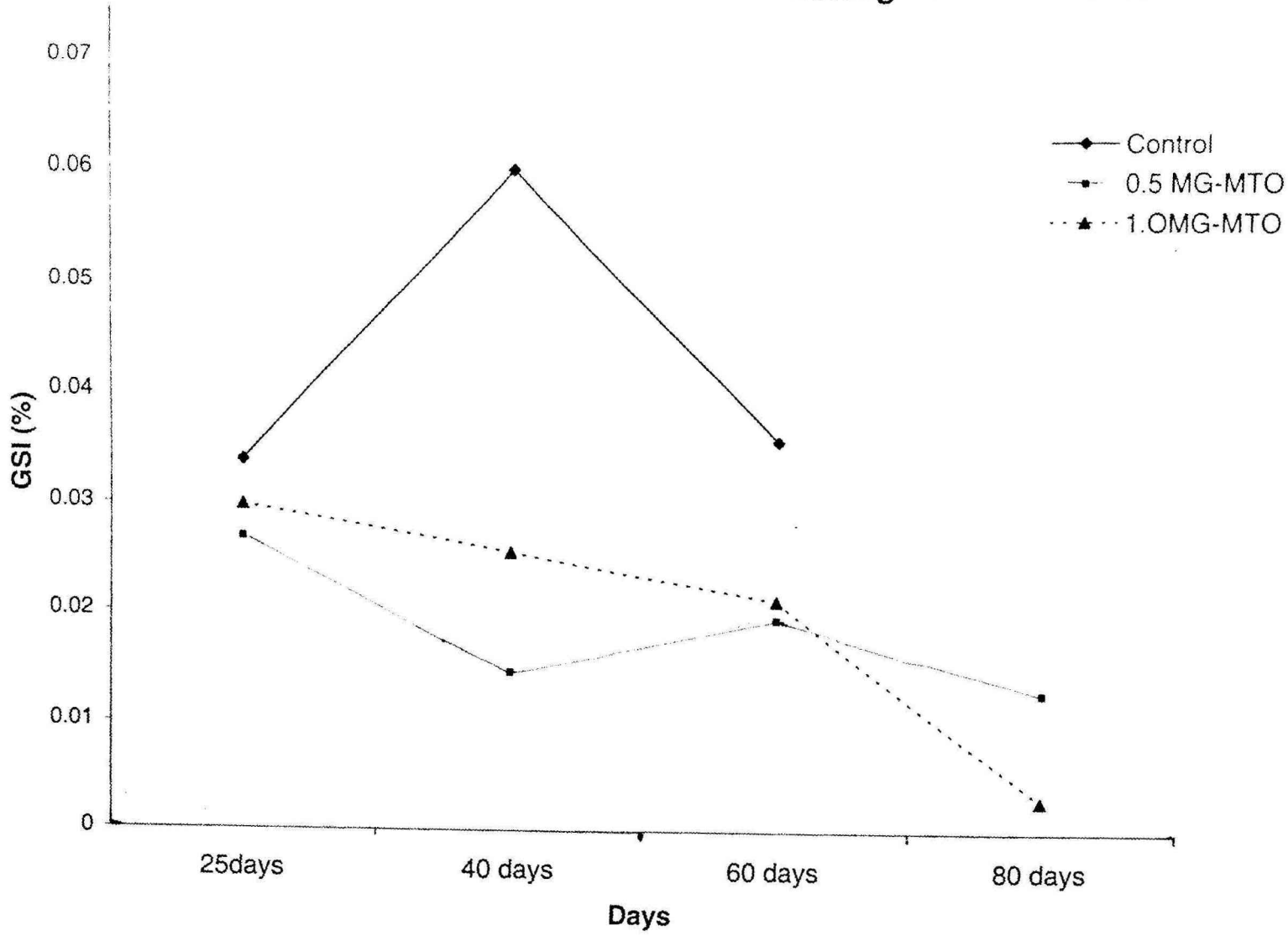
Plate 20 b. Degeneration of the 1.0MG-MTO gonad (280x) on 80th day
Gonadal class 8
SP-Spermatocytic activity

4.8 CHANGES OBSERVED IN THE GSI OF THE EXPERIMENTAL FISHES

Figure .1 depicts the GSI observed in the experimental fishes during the tenure of the experiment. The GSI of the fishes fed on the diet of Control – MTO was always higher than that of the MT fed fishes. The lower values of GSI observed in the hormone treated fishes in comparison to the values of the control group is an additional proof to show the progress of degeneration of the ovarian tissue and the initiation of the male tissues. It may also be reasonable to interpret that the 0.5 mg MT dose is better in comparison to 1.0 mg MT in terms of its efficacy in inducing sex reversal.

The GSI of the 0.5 MG-MTO remained lower than that of 1.0 MG-MTO till the 60^{th} day. However a slight increase in the GSI of the 0.5 MG-MTO compared to that of1.0 MG-MTO could be seen on the 80^{th} day in the graph.

Fig.1 Changes in GSI observed during MT treatments



5.DISCUSSION

5. DISSCUSSION

Aquaculture is increasingly recognized as a sustainable means of producing high quality aquatic foods for human consumption. In aquaculture as in agriculture there are areas where science plays an important role. These include reproduction, early development, nutrition, health and genetics (Donaldson, 1988). Among these reproduction is an important area, since reproduction in captivity has been the major key in aquaculture, which has opened the door for successful early rearing, metamorphosis and grow out to market size. One sub area in controlled reproduction of finfish, which is increasing in importance, is the regulation of sex."It thus becomes of great interest to discover the mechanism by which sex is determined and to find whether by any means we can bring it under control" (Huxley, 1938).

Smith (1959) had studied several members of the subfamily Epinephelinae and confirmed them as protogynous hermaphrodites based on the following observations: 1) No males were found in the smaller sized groups. 2) Males appeared among medium sized fish and the proportion increased among the higher sized classes until the larger sized groups were exclusively males.3) Regressive oocytes were found in the testes of the functional males.

In the present experimental study on *E.diacanthus* the wild and experimental fishes used were in the smaller length groups (125-217 mm SL) and all of them were confirmed females. It confirms the view that *E.diacanthus* is a protogynous hermaphrodite.

Tessy (1994) had made an extensive study on *E.diacanthus* from the south - west coast of India, for a period of two years (Dec '90 to Nov '92). She has recorded that majority of the smaller size group fishes mature as females and most of the males appeared from females after sex inversion. The observation of Tessy (1994) was also taken to confirm *E.diacanthus* as a protogynous hermaphrodite. In her study by examining the specimens caught from the wild she has concluded that immature stages of *E.diacanthus* occurred in length groups of 91-110mm. The selection of the fishes for experiments in the present work was based on the above study by Tessy (1994). In the present study the smallest size of the fish in which

primary developing oogonia were observed was 125 mm SL, which is slightly bigger compared to the observations by Tessy (1994) because of the lesser sample size chosen for the study in our work.

Sex control is also of importance for maintaining the economic efficiency of the production systems. In many species one sex grows faster, matures later or has a higher market value than the other sex. Monosex or sterile populations also serve specific culture purposes. Studies on the endocrine control of sex differentiation were initially conducted on ornamental fishes such as the Medaka, *Oryzias latipes* (Yamamoto, 1969). Since then, studies on a range of economically significant species have led to the utilization of sex control in commercial aquaculture (Hunter and Donaldson, 1983). Sex control is of particular importance in the culture of tilapias (Mc Andrew, 1993), Pacific salmonids, *Oncorhynchus sp* (Piferrer and Donaldson, 1993) and its utilization is being examined in other cultured species such as the carps (Cyprinidae), flatfish (Pleuronectiformes) and groupers (Serranidae) (Donaldson *et al.*, 1989). In recent years several of the protogynous groupers were also chosen as candidate species for aquaculture. Sex control and sex reversal can be brought about by the use of sex steroids (Yamamoto, 1969).

Research efforts have been directed at the induced breeding of the blue spotted grouper in particular, to solve the problem of establishing mature male broodstock in captivity. As the groupers are protogynous hermaphrodites, natural sex reversal through hormone therapy is therefore vital to grouper propagation and culture development (Kuo *et al.*, 1988).

The present study in *E.diacanthus* was done with the major objective of studying the actual process of reversal of sex in primary females by administering 17α- MT at 0.5 mg/kg BW and 1.0mg/kg BW orally. In this study initiation of the process of sex reversal could be seen on the 25th day. A complete reversal in the sex of the MT treated experimental fishes could be seen on the 40th day. The gonad continued to be in the state of degeneration on the 60th as well as on the 80th day of the experiment also.

In the present study on *E.diacanthus*, on the 25th day, gonads taken from the control fishes (125mm SL) appeared roundish and off white in colour on

external examination, while the treated gonads (0.5 MG-MTO and 1.0 MG-MTO) of *E. diacanthus* were shrunken and emaciated. The shrinkage was very drastic on the 40th day and it extended till the 80th of the experiment. However the control gonads stayed roundish and fleshy even on 40thand 60th days. On the 40 th day the removal of the treated gonads was very difficult. The surrounding viscera and the swim bladder had to be cleared to remove the treated gonads. Similar observations on the shrinkage of treated gonads had been recorded by Kramer *et al.* (1988) in their experiment on the bluehead wrasse, *Thalassoma bifasciatum* wherein the removal of gonads of testosterone treated fishes was very difficult "sometimes it was necessary to remove surrounding visceral masses, e.g., gut and spleen". This shrinkage may in part be contributed by the disappearance of oocytes through the process of degeneration.

Chen *et.al.*, (1977) reported that two of the 3 groupers of the species, *Epinephelus tauvina*, fed 80 mg MT over 30 days resulted in sex inversion. Subsequently all 25 fishes given a dosage of 1 mg MT/ kg diet, 3 times in a week over two month period underwent sex inversion. A very significant observation is that the dosage given in the present study was very low i.e., 9.9 mg MT/kg in 0.5 mg dosage and 19.1 mg MT/kg in 1.0 mg dosage in a period of 25 days compared to 80 mg MT during thirty days in *E.tauvina* by Chen *et al.* (1977).

In the present study on *E.diacanthus*, there was a drastic reduction in the gonadosomatic index of 0.5 and 1.0 mg treated fishes compared with that of the untreated experimental fishes. On the 40th day, the GSI of the control fishes were 0.0605 whereas the GSI of 0.5mg and 1.0mg treated fishes was 0.0148 and 0.0258 respectively. In 0.5mg hormone treated fishes size of the gonad had reduced by four times (0.0605/0.0148= 4) while in the 1.0 mg treated fishes the gonad had reduced twice (0.06065/0.0258=2) Such reduction in the GSI was recorded by Kuo *et al.*, (1988) while inducing sex reversal in the blue-spotted grouper *Epinephelus fario* by oral administration of 0.5 mg and 1.0mg methyl testosterone for a period of five months.

Kramer *et al.* (1988) studied in depth the effects of testosterone implants in the bluehead wrasse, *Thalassoma bifasciatum*. The untreated intersex gonad of *Thalassoma bifasciatum*, they observed were "small, firm to touch and

appeared white when viewed macroscopically. Histologically male and female germ elements were present. Oocytes were still abundant; however, they were at different stages of degeneration, as evident from the considerable variation in their size, shape and staining characteristics. The male germ cells were present as small aggregates or islands most often concentrated near the periphery of the lamellae. At no time was sperm present". Our histological observations in *E.diacanthus* intersex gonads obtained by feeding 0.5 mg and 1.0 mg MT around the 25th day are very similar to the observations by Kramer *et al.* (1988).

Surprisingly *Thalassoma bifasciatum* implanted with testosterone pellets showed degeneration of the ovarian tissue being initiated on the 21st day. However there was no evidence of spermatogenic tissue (as observed in the wild untreated intersex gonads). On the 40th day the degeneration of the treated gonad progressed further. However there were no signs of early or late spermatogenic tissue within the ovarian wall. Our observations in *E.diacanthus* are contradictory with that of Kramer, since we were able to observe spermatogenic tissue on the 25th day and more spermatogenic tissue occupying the entire gonad.

Kuo *et al.* (1988) in his study on the induced sex reversal of the blue-spotted grouper *Epinephelus fario*, observed that in a period of about, one month, the MT (0.5 mg and 1.0 mg) treated fishes were in their transitional stages. Degenerating oocytes could be seen histologically. Our observations on the intersex gonads on the 25th day of experimentally treated fishes are very similar to the observations of Chan and Yeung (1983) and Kuo *et al.*(1988). The processes of atresia of the ovarian tissue and a spurt spermatogenic activity were seen progressing in the treated gonads of *E.diacanthus* in the present study.

Chan and Yeung (1983) observed the process of spermatogenesis amidst the existing oocytes in the gonad of a natural sex reverting grouper *Epinephelus akaara*. Our observations on the gonads of the MT treated animals around the 25th day were similar to the above observation. However we have not studied the natural sex reversal in *E.diacanthus* in our experiments

Earlier attempts by Chan *et al.* (1975) to induce sex reversal through pellet implantation or multiple injections (Tang *et al.*, 1974) in the protogynous

Monopterus albus were unsuccessful. Detailed studies by Chan and Yeung (1983) over the years had been focussed on the endogenous hormone levels of the sex reverting females. Chan and Yeung (1983) finally postulated that the endogenous accumulation of the androgens was only a secondary event and not the actual cause for the real transformation of the ovary to testis. However our results were unlike the study of Chan and Yeung (1983), and evidence of the gonad reverting to maleness was very clear even on the 25th day. The degeneration of the gonad was observed on the 40th, 60th and on the 80th days. However to enlighten on the subject of functional maturity more detailed studies will have to be made.

Debas *et al.* (1989) undertook studies on the morphological and endocrinological aspects in a grouper *E.microdon*. He observed that sex inversion in *E. microdon looks* like a return to the immature female stage before the development of male gametogenesis. His work was based on the observations of the Polynesian grouper *E.microdon* maintained in tanks. This hypothesis appears to be partly true in this experiment on *E.diacanthus*, since on the 25th day, majority of the oocytes had regressed, somewhat confirming the observation by Debas *et al.* (1989).

Kuo *et al.* (1988) in their experiments on the blue-spotted grouper, *E.fario* fed the fishes with MT thrice a week at two different dosages 0.5 mg and 1.0 mg/kg BW of the fishes. His experiments resulted in functional males at the end of five months. In the present study, sex inversion of female to male was achieved in a period of 40 days. A very notable similarity between the observation of Kuo *et al.* (1988) and our observation was the occurrence of degenerative changes: observed by Kuo *et al.*, on the 40 th day with the reversing gonads of *E.fario* and observed by us on the 60th and 80th days in *E.diacanthus*.Kuo *et al.*, observed functional maturity around 145 days wherein the treated gonads had spermatids and spermatozoa. However we terminated our experiment on the 80th day and we did not have functional maturity of gonads taking place.

One of the significant findings in our experiment is that even a very low dosage of androgen can induce sex reversal in a protogynous hermaphrodite. However our observations on the influence of androgens on the sex reversal as well as observations of earlier stalwarts such as Chen *et al* .(1977) , Kuo *et el.*(1988) on other species of groupers such as *E.tauvina* and *E.fario* respectively, should not be

taken as a sole method of obtaining males for fertilization in the protogynous teleosts; especially in the event of ban on the usage of androgens by some developed nations. We should think of alternative methods of sex reversal, taking into consideration the basic reasons for sex inversion such as endocrinological aspects (Yamamoto, 1962), social aspects (Robertson, 1972) etc. However experiments, as the one that we have initiated would certainly enable us to understand many other mysteries inherent in this process of sex inversion and the enigma of hermaphroditism.

SUMMARY

SUMMARY

E. diacanthus, a protogynous hermaphroditic grouper was used as a model fish to study the influence of the androgen, 17α-Methyl testosterone (MT) on the reproductive physiology mainly with reference to sex reversal.

Prior to conducting actual experiments to achieve the main objective, detailed studies in relation to the maturity stages of wild fish were conducted. These studies proved that *E. diacanthus* is a protogynous fish.

For the experimental study on the influence of MT, three groups of fishes of 12 numbers were reared in similar rearing containers of 5 ton capacity provided with inbuilt biofilters. One of them was used as a control and the fishes were fed with a formulated diet without MT. Group two and three were fed with 0.5mg and 1.0 mg MT/kg body weight incorporated in formulated diets. Fishes were fed at 2% of their body weight. The fishes were reared for a period of 80 days. The special diet formulated for the experiment was observed to be eagerly accepted by the groupers.

Periodically (25th day, 40th day, 60th day and 80th day of experiment) representative samples of fish from each group were sacrificed and their gonads subjected to detailed morphological and histological studies. The detailed data collected from the experimental fishes show that feeding of methyl testosterone was accompanied by a drastic shrinkage of the gonads. Gonads of control fishes remained roundish and bulgy. The shrinkage of the gonad of the experimental fishes continued till the 80th day of the experiment.

The GSI of the 1.0 mg MT fed fishes decreased gradually from 0.03 on the 25th day to 0.0258 on the 40th day. Further decrease in the GSI was very drastic. The GSI of the 0.5 mg MT fed fishes also decreased from 0.027 on the 25th day to 0.0148 on the 40th day. The GSI of the 0.5 mg showed a slight increase on the 60th day and again decreased on the 80th day. The GSI values of the control fishes were always above that of the MT fed fishes.

The detailed changes in the histology of the gonads in MT treated and untreated gonads were studied in detail. Initiation of the process of sex reversal with the gonads reverting to maleness could be seen on the 25th day in the MT treated fishes. Both the dosages initiated sex reversal and were equally effective. Sex reversal was complete by 40th day.

Degeneration of the ovarian tissue continued from the 25th day till the end of the experimental period. Spermatogenic activities continued in the degenerating gonad till the 80th day.

Inspite of the sex reversal, functional maturity of the testes did not take place in androgen fed fishes even on the 80th day.

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